Separation of the 2,4-Dinitrophenylhydrazones of Dicarbonyl and Other Polar Compounds by Liquid-Liquid Partition Chromatography

▶ **In order to investigate some highly polar carbonyl compounds found in oxidized whole milk powder, suitable liquid-liquid partition chromatographic systems for separating the mono- and bis(2,4 - dinitrophenylhydrazones) of these carbonyl compounds were devised. Three column systems are described, each one suited to handle a different range of compound polarities. Mixtures of acetonitrile and water are used as the stationary phase on Celite columns. Elution is done with methylcyclohexane or methylcyclohexane–ethyl acetate mixtures. Monocarbonyl, bis(oxoaldehyde) and diketone bis(2,4-dinitrophenylhydrazones) have been studied.**

DURING A STUDY of the carbonyl compounds associated with the oxidized flavor of whole-milk powder there was obtained a variety of dicarbonyl and highly polar monocarbonyl 2,4-dinitrophenylhydrazones (2,4-DNPH's). Methods in the literature for separating these derivatives (*4, 5*) were inapplicable, and a previously published method for monocarbonyl 2,4-DNPH's by the author and others (*1*) was not suited for these polar derivatives. A liquid-liquid partition chromatographic column was considered the most desirable method of separating these compounds. Three partition columns have been evolved for this purpose since it was found that one partition system could not handle the wide range of polarities encountered.

EXPERIMENTAL

Reagents and Apparatus. Spectrophotometer to accommodate optically matched test tubes (15 ml. or larger). Chromatographic tube, approximately 25 × 350 mm. with solvent reservoir.

Methylcyclohexane. Redistill and collect the 101° to 103° C. fraction.

Acetonitrile. Redistill and collect the 80° to 82° C. fraction.

Ethyl Acetate. Redistill and collect the 77° to 78° C. fraction.

Dry Column Materials. Analytical grade Celite (Johns-Manville analytical filter aid) and alumina (80 to 200 mesh, for chromatographic analysis, Fisher Scientific Company) were dried in a 140° to 150° C. oven for at least 24 hours before use.

Column Preparation and Use. COLUMN 1. An equilibrated eluting solvent is prepared by saturating methylcyclohexane with acetonitrile at room temperature. Fifteen grams of dry Celite are placed in a blending container with 200 ml. of the equilibrated methylcyclohexane and mixed until wetted. In a graduated cylinder 0.3 ml. of water and 12 ml. of acetonitrile are mixed and then poured as a fine stream into the swirling Celite slurry. When homogeneous, the slurry is poured through a wide-stem funnel into the column, the tip of which has been closed with a pinchcock. Air bubbles are removed with a tamping rod. The pinchcock is opened and the Celite is compacted with 2 to 4 p.s.i. air pressure. The top of the column, which is always kept covered with solvent, is firmed and leveled with a tamping rod.

COLUMN 2. The eluting solvent in this case is 2% ethylacetate in methylcyclohexane (v./v.). This solvent is then equilibrated by saturating it with acetonitrile at room temperature. Fifteen grams of dry Celite and 200 ml. of the equilibrated solvent are mixed as before, but only 6 ml. of acetonitrile containing 0.3 ml. of water are added to the swirling Celite slurry. The remainder of the column preparation is the same as for column 1.

COLUMN 3. An eluting solvent is prepared by mixing 6% ethylacetate in methylcyclohexane (v./v.) and then saturating this mixture with acetonitrile at room temperature. A small layer of Celite deficient in stationary phase is needed at the bottom of column 3. Its function will be explained in the Discussion. Two grams of dry Celite and 60 ml. of equilibrated solvent are mixed by swirling in a tall beaker and then poured into the closed chromatographic tube. After settling slightly, the tube is opened and the Celite is compacted with air pressure. At least 2 to 3 cm. of solvent are allowed to remain above this layer to prevent its being disturbed when the column proper is made on top of it. Fifteen grams of dry Celite and 6 grams of dry alumina are mixed with 200 ml. of the equilibrated eluting solvent. Ten milliliters of acetonitrile and 0.4 ml. of water are mixed and then poured as a fine stream into the swirling Celite–alumina slurry. When homogeneous the slurry is poured carefully into the tube so as not to disturb the lower Celite layer. Air bubbles are removed, air pressure is applied, and the column is finished in the same manner as column 1 or 2.

The partition columns are operated in the usual manner taking into account the following suggestions and limitations. The 2,4-DNPH's are dissolved in the proper equilibrated eluting solvent depending upon which column is to be used. A small volume, 10 ml. or less, is most desirable but as much as 20 to 25 ml. can be used if necessary. If any insoluble material is present, it must be filtered out before putting the sample on the column. Flow rate to start should be about 60 drops per minute. When the color starts to move the partition properly, the flow rate can be increased to as much as 150 drops per

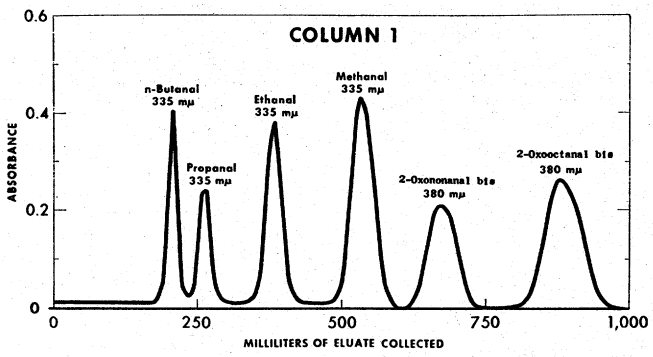

Figure 1. Separation of 2,4-dinitrophenylhydrazones on column 1

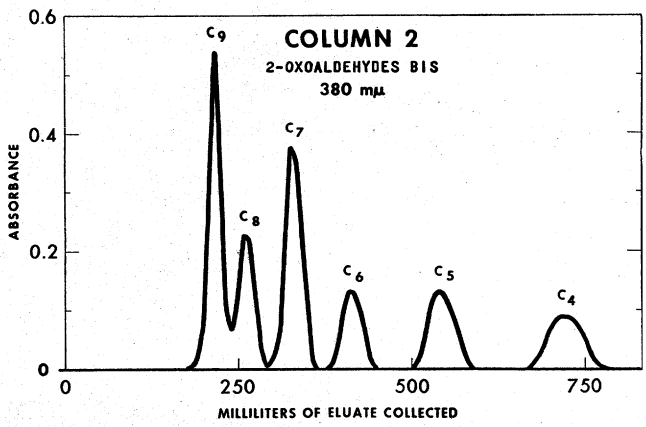

Figure 2. Separation of the bis(2,4-dinitrophenylhydrazones) of 2-oxoaldehydes on column 2

minute. Column eluate is collected in fractions of known volume from the time the sample is put on the column and the absorbance of each fraction is measured in the spectrophotometer. Fresh equilibrated eluting solvent or clear column eluate can be used as the reference solution for the spectrophotometric measurements. Blank readings, if present at all, will be small but should be allowed for where quantitative values are wanted. Largest blank readings will be found when measuring monocarbonyl derivatives in the 335- to 340-mμ range.

Dissolving the polar 2,4-DNPH's in the desired eluting solvent can be difficult at times. If solution does not take place quickly to give the desired concentration of color, frequent agitation of the flask for an hour or so may help. Time is a necessary factor in dissolving some of these polar crystals. Evaporation of the more volatile ethyl acetate and acetonitrile should be prevented as these two components in the eluting solvent contribute most to its dissolving power. For some highly insoluble compounds to be separated on column 3 a special dissolving solvent is made up by mixing 15% ethyl acetate with methylcyclohexane (v./v.), and then saturating this mixture with acetonitrile at room temperature. Up to 25 ml. of this solution can be used to dissolve the sample to put it on the column, but eluting is then done with the regular column 3 eluting solvent. This special dissolving solvent is needed for the bis(2,4-DNPH's) of glyoxal, methylglyoxal, diacetyl, and others of similar strong polarity. Using a stronger solvent in this manner to put a sample on the column is not good partition chromatography technique as it is sure to disrupt the column slightly, but in the case of column 3, the return of the column to normal partitioning occurs very quickly.

RESULTS AND DISCUSSION

Figures 1, 2, and 3 show the type of separations to be expected from each of the columns as well as the kind of 2,4-DNPH's which each is capable of resolving. The wavelength at which each compound is measured is also given. Table I summarizes the data on all the 2,4-DNPH's.

Column 1. This column is of value when separating the more polar 2,4-DNPH's of monocarbonyl compounds. These are the derivatives which, in the usual system for separating monocarbonyls, are the last to be removed from a column and require the collection of a large number of eluate fractions. The methylcyclohexane–acetonitrile system of column 1 separates these derivatives quickly and sharply. Bis(2,4-DNPH's) of dicarbonyl compounds of eight or more carbon atoms are also separated on this column. When dealing with an homologous series of 2,4-DNPH's, overlapping of peaks is no problem, except for the first few fast running bands, but when a mixture of derivatives from two or more homologous series is chromatographed, overlapping of peaks is certain to occur. Overlapping peaks can be separated eventually by rechromatographing the mixture on a column containing $1\frac{1}{2}$ to 2 times as much stationary phase, or rechromatographing them on an entirely different column. Column 1 lends itself to the first method because it is not loaded to capacity with stationary phase, and the amount of acetonitrile can be increased at the discretion of the user.

Column 2. This column takes up where column 1 leaves off; that is, the 2,4-DNPH's which come off last on column 1 become the first, fast moving bands when put through column 2. This makes it ideally suited for separating the bis(2,4-DNPH's) of dicarbonyl compounds of intermediate size and polarity such as the C_{10} to C_5 derivatives. C_4 compounds may or may not move satisfactorily on column 2. Column 2 uses an eluting solvent that contains ethyl acetate, methylcyclohexane, and acetonitrile, all in equilibrium with each other. Because its composition is easily altered by temperature changes and evaporation, these should be kept to a minimum. Overlapping of peaks will occur on column 2 also when derivatives of two or more homologous series are chromatographed at the same time. Overlapping peaks can usually be separated by rechromatographing them on a column 1. Column 2 is also underloaded with stationary phase; therefore, the amount of acetonitrile on the column can be increased if a particular problem indicates that such an increase would be helpful.

Column 3. This column was designed to accommodate the more polar dicarbonyl derivatives which refuse to move easily on column 2. It uses an eluting solvent similar to column 2 but much stronger (6% ethyl acetate). Column 3 is prone to washing or a movement of the stationary phase down, and off, the column material. This is due to the polar nature of the eluting solvent. If stationary phase is allowed to wash off the column, a turbid eluate is produced the color of which cannot be measured in the spectrophotometer. To prevent this from happening, a zone of 2 grams of Celite, deficient in stationary phase, is placed below the column proper as described in the column preparation section. This washing of stationary phase is easily seen at the top of the column where a darker zone forms and moves slowly down the column, but because the colored bands move and partition ahead of this zone, it does not interfere with the operation of the column. The support material used in column 3 is a mixture of Celite and alumina. The purpose of the alumina is not to make an adsorption type column, but to give added stability to the stationary phase. Some adsorption undoubtedly takes place however. Because a large amount of ethyl acetate passes through column 3, a support with more adsorptive capacity than Celite is needed. The type of alumina described in the reagent section possesses the proper adsorptive qualities without unduly affecting the symmetry of the colored bands or disrupting the partitioning qualities of the Celite column.

Peak Elution Volume. The number of milliliters of eluting solvent required to elute the fraction of deepest color for each derivative chromatographed is called the peak elution volume. In partition chromatography it is a constant and characteristic value so long as all columns prepared are identical in composition and partitioning characteristics. Under practical conditions of analysis it is usually found that the peak elution value will vary from column to column through some range of values. Three variables that can easily affect the peak elution volumes are: a variation in the temperature at which the column is prepared and operated; a variation between different batches of Celite; and excessive eluting speed. Variations in the peak elution volume values were found with the three columns studied. The volumes given in Table I are average values to be used for the tentative location of the various derivatives. Final identification of an unknown 2,4-DNPH must be based on observations other than the peak elution volume.

Recovery Values. One advantage of using a partition chromatographic column is that 100% recovery of added material is easily obtained. Such is the case with columns 1 and 2 where recoveries were consistently high and for most purposes can be considered 100%. Recoveries on partition columns can vary, however; van Duin (*3*) reports 98 to 100% recoveries on a neutral and an acid silica gel column, but on another acid type column recovery values ranged from 0 to 100%, the loss being attributed to acid catalyzed decomposition. The longer the material remained on the column, the greater the decomposition and the lower the recoveries. A similar situation exists with column 3, where the fast moving zones give high recovery values and the slow moving zones give lower recoveries. Recovery values will also vary on column 3 with the amount of material put on; the more color put on, the higher is the percentage recovery. For these reasons no values are given in Table I for the slow moving bands on column 3 and they should be considered qualitative in nature until recovery measurements have been made for a particular derivative under study.

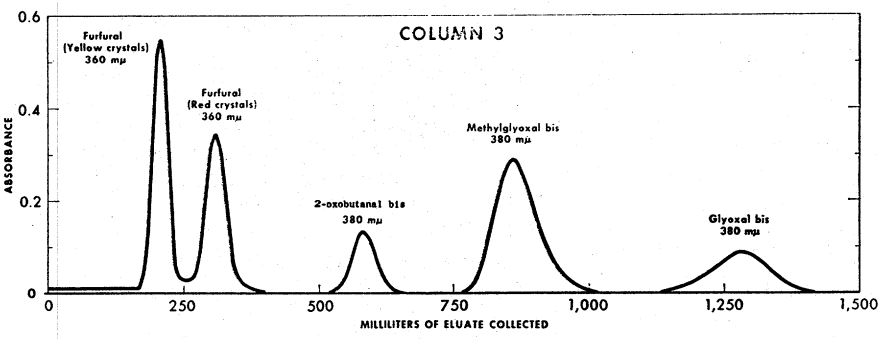

Figure 3. Separation of 2,4-dinitrophenylhydrazones on column 3

Solvents and Equilibration. All solvents are distilled to remove traces of nonvolatile constituents. These nonvolatile compounds do not interfere noticeably with the operation of the columns but are undesirable when the 2,4-DNPH crystals are wanted for further study.

The equilibration of the eluting solvents at the same temperature as the column is necessary for optimum results. The mixing of methylcyclohexane, ethylacetate, and acetonitrile is an endothermic process, the final solution being 3° to 5° C. cooler than the starting solvents. Establishing the final room temperature equilibrium from this cool side is desirable because it assures a clear supernatant solvent. If the solvent should go above room temperature and then cool a degree or two, a fine cloudiness results which will not clear up except by adding some new solvent mixture and approaching equilibrium again from the cool side. An undissolved globule of acetonitrile should be visible in the bottom of all equilibrated solvent flasks.

At times a cloudiness, or small droplets of stationary phase, may be seen in some of the eluate fractions. If there is color in these fractions and it is desired to measure it in the spectrophotometer, the cloudiness or droplets can be dissolved by adding sufficient ethyl acetate to each fraction to give a homogenous solution. The amount needed is usually so small that the reading will not be affected. The Celite and alumina used as support material are dried in a hot air oven so that the amount of moisture incorporated when making up each column can be kept uniform. A correct moisture content in the columns makes them easier to compress and compact, it helps to hold the acetonitrile in place, and it helps in separating the colored zones. This last aspect, of aiding in the separation, is reported in detail by van Duin (*2*, *3*) and is called by him liquid-liquid interface

Table I. Summary of 2,4-Dinitrophenylhydrazones Studied

Parent Compound of 2,4-DNPH's	Chromatographed on Column 1	2	3	Peak Elution Volume, ml.	Color Measured at mμ	Recovery, %
n-Butanal	×			215	335	97
Propanal	×			265	335	100
Ethanal	×			380	335	104
Methanal	×			555	335	104
Acetone	×			250	345	100
2,4-Dinitrophenylhydrazine			×	760	335	
Bis(2-oxononanal)	×			680	380	
Bis(2-oxononanal)		×		235	380	99
Bis(2-oxooctanal)	×			890	380	
Bis(2-oxooctanal)		×		285	380	98
Bis(2-oxoheptanal)		×		345	380	96
Bis(2-oxohexanal)		×		445	380	96
Bis(2-oxopentanal)		×		555	380	97
Bis(2-oxobutanal)		×		755	380	97
Bis(2-oxobutanal)			×	620	380	82
Bis(2,3-octanedione)		×		235	380	97
Bis(2,3-heptanedione)		×		310	380	99
Bis(2,3-pentanedione)		×		520	380	
Bis(2,3-pentanedione)			×	560	380	88
Bis(Diacetyl)			×	630	380	
Bis(Methylglyoxal)			×	860	380	
Bis(glyoxal)			×	1360	380	
Furfural (yellow crystals)	×			300	360	
Furfural (yellow crystals)		×		180	360	100
Furfural (yellow crystals)			×	205	360	100
Furfural (red crystals)	×			770	360	
Furfural (red crystals)		×		405	360	100
Furfural (red crystals)			×	310	360	100
Crotonaldehyde	×			275	355	98
Mesityl oxide	×			130	360	98
Acrolein	×			300	350	
Acrolein		×		205	350	100

adsorption. It can proceed at the same time and on the same column with liquid-liquid partitioning. It is a mild type of adsorption and occurs when a partition column is not loaded to capacity with stationary phase.

The 2,4-DNPH's that can be separated or purified on the three columns are not limited to those listed in Table I. Any 2,4-DNPH that has a polarity between the *n*-butanal derivative and the bis derivative of glyoxal should partition on one of the three columns.

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LITERATURE CITED

(1) Corbin, E. A., Schwartz, D. P., Keeney, Mark. *J. Chromatog.* **3**, 322 (1960).
(2) Duin, H. van, *Nature* **180**, 1473 (1957).
(3) Duin, H. van, Reports from the Netherlands Institute for Dairy Research (N.I.Z.O.), No. **54**, May (1961).
(4) Meigh, D. F., *Chem. Ind. (London)* **1956**, 986.
(5) Wolfrom, M. L., Arsenault, G. P., ANAL. CHEM. **32**, 693 (1960).

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